Remarks

Applicants request entry of the amendments. The amendments place the application in condition for allowance or better form for appeal.

Applicants have amended the specification at page 12 as suggested by the Examiner. One of skill in the art was familiar with the 72K protein of the E2A region of adenovirus as one of the proteins encoded by E2, and clearly the word "especially" in this paragraph identifies that other regions of E2 exist and can be selected for use in the cell lines of the invention.

Applicants have amended claims 36, 45, and 61 to recite particular gene or adenoviral regions as "the only adenoviral genes that have been rendered non-functional." These amendments address the Examiner's comments at pages 3-4 of the Office Action. Furthermore, claim 45 includes the Examiner's suggested language with respect to the optional E3 gene.

Claim 73 is amended to change the recitation of E3 to E2.

Applicants have added new claim 85, as required to preserve the subject matter according to the Office Communication of April 10, 2006.

No new matter enters by these amendments.

Response to Rejections

Claims 36-39 and 45-84 stand rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the enablement requirement. The Examiner suggests that embodiments where specific adenoviral genes are the only non-functional genes present can be enabled.

Applicants' specification refers to many known and characterized adenoviral regions (*see* page 2, lines 1-20, for example). The specification also specifically indicates that non-functional E1, E2, E4, L1-L5 can be used (*see* page 3, line 34, through page 4, line 5, for example). The specification also specifically refers to all of part of E2 and all or part of E4. The Examples in the specification also detail the numerous replication defective adenoviruses and complementing cell lines one of ordinary skill in the art could produce from the teachings of Applicants' specification. One of skill in the art at the time could have used the available information on adenoviral sequences to prepare replication defective

adenoviruses and complementing cell lines containing multiple deletions, mutations, or non-functional regions. In fact, one of skill in the art knew of existing adenoviral mutants containing deletions that one of skill in the art could have used for these purposes (*see* Thimmappaya et al., enclosed).

Furthermore, the specification discusses the use of complementing cell lines, including 293 cells, KB cells, HeLa cells, for example, at page 11, line 35 through page 12, line 1, and page 15, lines 12-30.

Accordingly, Applicants respectfully submit that the specification, coupled with the skill one in the art would have possessed, would allow the introduction of more than a single or a specified number of gene modifications. The ability to manipulate adenoviral sequences to effect function was known, for example in Le Gal La Salle et al. (Science 259:988-990; copy enclosed). In combination with the teachings of this specification, numerous regions and genes within the adenoviral genome could have been rendered non-functional to produce replication defective adenoviruses, as well as cell lines that complement the non-functioning genes.

Furthermore, in accordance with the reasoning in the unpublished decision of Ex parte Saito (copy enclosed), and the Federal Circuit precedent in In re Cortright, 49 U.S.P.Q.2d 1464 (Fed. Cir. 1999) and CFMT, Inc. v. Yieldup Int'l Corp., 68 U.S.P.Q.2d 1940 (Fed. Cir. 2003), it appears that the Examiner is requiring that an improper burden be met to show enablement in this case. Applicants need not show that every conceivable embodiment of the claims be operable. Also, Applicants need not show that aspects of the claims that are not recited be specifically enabled. In this case, the apparent reasoning is that claims cannot be enabled unless there is a specific, enabling demonstration that what they do not recite (i.e., non-functional genes not recited in the claims). Applicants submit that such a burden is inappropriate. Applicants have stated in the specification that the invention as claimed is enabled and provided sufficient examples and description to support the claims.

Solely to advance prosecution, Applicants have added to claims 36, 45, and 61 a recitation that relates to only particular adenoviral genes being rendered non-functional.

The Examiner rejects claims 73-76 under 35 U.S.C. § 112, first paragraph, for failure to comply with the written description requirement. Applicants have amended claims 73 to recite "E2" instead of "E3." The specification describes such cell lines at, for example, page 12, lines 5-13, and the Examples.

As all the objections and rejections have been addressed and countered by argument or amendment, the application is in condition for allowance. Timely notification of allowability is requested.

If there are any additional fees due with the filing of this document, including fees for the net addition of claims, applicants respectfully request that any and all fees be charged to Deposit Account No. 50-1129. If any extension of time request or any petition is required for the entry of this paper or any of the accompanying papers, applicants hereby petition or request the extension necessary. The undersigned authorizes any fee payment from Deposit Account No. 50-1129.

Respectfully submitted,

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UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Ex parte NORIMITSU SAITO and MING ZHAO

Appeal No. 2005-1442
Application No. 09/734,786

ON BRIEF

Before ELLIS, SCHEINER, and GRIMES, Administrative Patent Judges.

GRIMES, Administrative Patent Judge.

DECISION ON APPEAL

This appeal involves claims to a method of introducing a nucleic acid into a subject by modifying and transplanting hair follicles. The examiner has rejected the claims as nonenabled. We have jurisdiction under 35 U.S.C. § 134. Because the examiner has not shown that undue experimentation would have been required to practice the claimed method, we reverse.

Background

The specification discloses that "histocultured tissues, including tissues containing hair follicles, can be successfully modified genetically ex vivo and then transplanted successfully into an intact mammalian subject. The success of the

modification is enhanced by treating the histocultured tissues with collagenase prior to genetic modification." Pages 2-3.

The specification states that

> [a]lthough it is advantageous to treat the cultured tissue with collagenase in order to enhance the ability of the tissue to accept heterologous nucleic acids, the treatment is not so severe as to destroy completely the integrity of the three-dimensional array.
>
> The three-dimensional histoculture can be assembled from any tissue, including skin, especially skin containing hair follicles, lymphoid tissue, or tumor tissue. The choice of tissue will depend on the nature of the treatment contemplated. . . .
>
> For example, hair follicles are useful recipients of genes intended to affect the growth or quality of hair, but also are able to produce immunogens and other products that may be useful to the organism taken as a whole.

Page 4.

The specification provides a working example in which DNA encoding green fluorescent protein (GFP) was introduced into hair follicles of histocultured mouse skin; the percentage of GFP-expressing hair follicles ranged from 22% to 67%. See pages 11-12. In a second working example, hair follicles in skin samples were transfected with GFP-encoding DNA and grafted onto recipient mice. The results showed that "the percentage of hair follicles with GFP fluorescence in collagenase-treated skin was 5.7 times greater than in hair follicles of untreated skin." Pages 14-15. Fluorescence was detected for at least 10 days after grafting. Figure 3B.

> [d]ue to the art recognized unpredictability of achieving therapeutic levels of gene expression following direct or indirect administration of nucleic acids and the lack of guidance provided by the specification for the parameters affecting delivery and expression of therapeutic amounts of DNA into the cells using ex vivo gene transfer into histocultured organs or tissues, it would require undue experimentation to practice the instant invention.

Examiner's Answer, page 10

Appellants argue that the claims are directed to a method of genetically modifying tissues ex vivo and transplanting the modified tissue into a subject, and therefore do not require achieving therapeutic levels of gene expression. Appeal Brief, page 5. Appellants point to the specification's discussion of prior art techniques and working examples as guidance to those skilled in the art. Appellants assert that "[t]he pending claims are fully supported by the ample amount of knowledge available in the relevant art when the present application was filed and the guidance provided in the specification." Id., page 7.

We agree with Appellants that the examiner has not adequately shown that undue experimentation would have been required to practice the claimed method. The examiner bears the initial burden of showing that a claimed invention is nonenabled. See In re Wright, 999 F.2d 1557, 1561-62, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993) ("[T]he PTO bears an initial burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by that claim is not adequately enabled by the description of the invention provided in the specification of the application.").

"[T]o be enabling, the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without 'undue experimentation.'" In re Wright, 999 F.2d 1557, 1561, 27 USPQ2d 1510, 1513 (Fed.

Discussion

1. Claim construction

Claims 1 and 11 are representative of the claims on appeal and read as follows:

1. A method to introduce a nucleic acid molecule into a mammalian subject which method comprises

transplanting into the dermis of said subject at least one hair follicle that has been modified ex vivo to contain said nucleic acid molecule.

11. A method to introduce a nucleic acid molecule into a mammalian subject which method comprises transplanting into the corresponding tissue of said mammal a histocultured intact tissue that has been modified ex vivo to contain said nucleic acid molecule;

wherein said histoculture has been treated with collagenase prior to modifying said tissue with the nucleic acid.

Thus, claim 1 is directed to a method of introducing a nucleic acid into a mammal by modifying a hair follicle ex vivo to contain the nucleic acid and transplanting the hair follicle to the mammal. Claim 1 does not explicitly require that the nucleic acid be expressed or provide any particular benefit to the mammal.

Claim 11 is similar to claim 1 but encompasses treating tissues other than hair follicles; in addition, claim 11 requires that the tissue be treated with collagenase before being modified with the nucleic acid.

2. Enablement

The examiner rejected claims 1-8, 11, 13-15, 17, and 19, all of the claims remaining, under 35 U.S.C. § 112, first paragraph, on the basis that the specification does not enable those skilled in the art to practice the claimed method without undue experimentation. The examiner considered the factors set out in In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988), and concluded that

Cir. 1993). "That some experimentation may be required is not fatal; the issue is whether the amount of experimentation required is 'undue.'" In re Vaeck, 947 F.2d 488, 495, 20 USPQ2d 1438, 1444 (Fed. Cir. 1991).

The enablement analysis must be focused on the product or method defined by the claims. "Title 35 does not require that a patent disclosure enable one of ordinary skill in the art to make and use a perfected, commercially viable embodiment absent a claim limitation to that effect." CFMT, Inc. v. Yieldup Int'l Corp., 349 F.3d 1333, 1338, 68 USPQ2d 1940, 1944 (Fed. Cir. 2003).

Here, the examiner has acknowledged that the claims are not limited to therapeutic methods, but argues that because therapeutic methods are encompassed by the claims, such methods must be enabled in order for the full scope of the claims to be enabled. See the Examiner's Answer, page 12.

The examiner's reasoning is logical but not entirely consistent with the case law: enabling the "full scope" of a claim does not necessarily require enabling every embodiment within the claim. See, e.g., Atlas Powder Co. v. E.I. Du Pont De Nemours & Co., 750 F.2d 1569, 1576, 224 USPQ 409, 414 (Fed. Cir. 1984): "Even if some of the claimed combinations were inoperative, the claims are not necessarily invalid. . . . Of course, if the number of inoperative combinations becomes significant, and in effect forces one of ordinary skill in the art to experiment unduly in order to practice the claimed invention, the claims might indeed be invalid." Atlas Powder concerned claims to a product, not a method as here, but the same principle applies – a claimed method does not lack enablement merely because it cannot be practiced under some circumstances or to achieve some particular result.

In re Cortright, 165 F.3d 1353, 49 USPQ2d 1464 (Fed. Cir. 1999), is instructive. In Cortright, the applicant claimed a method of "treating scalp baldness with an antimicrobial to restore hair growth." Id. at 1355, 49 USPQ2d at 1465. The Board reversed a rejection for lack of utility, but entered a new rejection for lack of enablement, on the basis that "restor[ing] hair growth" required returning the user's hair to its original state (a full head of hair). See id. "Because Cortright's written description discloses results of only 'three times as much hair growth as two months earlier,' 'filling-in some,' and 'fuzz,' the board reasoned, it does not support the breadth of the claims." Id. at 1358, 49 USPQ2d at 1467.

The court disagreed with the Board's claim interpretation, holding that "one of ordinary skill would construe this phrase [restoring hair growth] as meaning that the claimed method increases the amount of hair grown on the scalp but does not necessarily produce a full head of hair." Id. at 1359, 49 USPQ2d at 1468. The court concluded that the claims, so construed, were enabled. Id.

As with the present claims, the claims in Cortright encompassed a method of obtaining results that might be difficult to achieve: here, therapeutically effective gene therapy; in Cortright, complete restoration of hair growth. However, as in Cortright, the present claims do not require that particular result: the present claims require only introducing or delivering a nucleic acid; Cortright's claims required only some restoration of hair growth.

The court in Cortright did not dispute the Board's conclusion that completely restoring hair growth using Bag Balm® would require undue experimentation. See id. at 1357, 49 USPQ2d at 1467. The court nonetheless concluded that the claimed method

was not nonenabled merely because it encompassed one difficult-to-achieve outcome. The same reasoning applies here: the examiner may be correct that achieving clinically useful gene therapy using the claimed method would require undue experimentation, but the claims are not nonenabled merely for encompassing that difficult-to-achieve outcome.

The claims are directed to methods of introducing a nucleic acid into a mammalian subject or delivering a nucleic acid to a hair follicle or intact tissue. The examiner has not adequately explained why the specification does not enable those skilled in the art to introduce a nucleic acid into a mammalian subject, or deliver a nucleic acid to a hair follicle or intact tissue, without undue experimentation. We therefore reverse the rejection for nonenablement.

REVERSED

Joan Ellis Administrative Patent Judge	)
Toni R. Scheiner Administrative Patent Judge	) BOARD OF PATENT) APPEALS AND) INTERFERENCES
Eric Grimes Administrative Patent Judge	)

EG/dym

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An Adenovirus Vector for Gene Transfer into Neurons and Glia in the Brain

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The efficient introduction of genetic material into quiescent nerve cells is important in the study of brain function and for gene therapy of neurological disorders. A replication-deficient adenoviral vector that contained a reporter gene encoding β-galactosidase infected rat nerve cells in vitro and in vivo. β-Galactosidase was expressed in almost all sympathetic neurons and astrocytes in culture. After stereotactic inoculations into the rat hippocampus and the substantia nigra, β-galactosidase activity was detected for 2 months. Infected cells were identified as microglial cells, astrocytes, or neurons with anatomical, morphological, and immunohistochemical criteria. No obvious cytopathic effect was observed.

The ability to deliver foreign genes and promoter elements directly to terminally differentiated cells of the nervous system, which no longer proliferate, would be desirable for the study of the function and regulation of cloned genes as well as for gene therapy. Although a possibility is offered by defective herpes simplex virus vectors (*1*), their usefulness has been limited by their poor efficiency of infection and their pathogenicity. Here, we show that adenovirus, whose natural target is not the nervous system but the respiratory epithelium (*2*), has the ability to infect nerve cells. The gene transfer and expression of adenovirus are highly efficient both in vitro and in the intact rat brain.

In addition to nonreplicative infection, adenovirus has several assets (*3*). Its genome can accommodate foreign genes of up to 7.5 kb. It has a large host range and low pathogenicity in humans, and high titers of the virus can be obtained (*4*). We used a replication-defective adenovirus, Ad.RSV-βgal, which expressed a nuclearly targeted β-galactosidase (β-gal) cDNA under control of the Rous sarcoma virus long terminal repeat (RSV LTR) promoter (*5*). We tested the ability of this vector to infect primary cultures of sympathetic neurons of superior cervical ganglia (SCG). These cells, cultured in the presence of an antimitotic agent, provided a pure and homogeneous preparation of neurons (*6*). After inoculation of the virus, virtually all cells were positive for β-gal activity (*7*), with no apparent toxic effects or morphological changes (Fig. 1, A and B). Labeled cells were not detected when the staining reaction was performed on a parallel, noninoculated culture (Fig. 1C). We also tested the ability of adenovirus to infect primary cultures of rat hippocampal tissue that were enriched in astrocytes (*8*). Inoculation resulted in a blue nuclear staining in about two-thirds of the cells (Fig. 1D). The identification of stained cells as astrocytes was confirmed by additional staining with an antibody against glial fibrillary acidic protein (GFAP) (Fig. 1E).

We next evaluated the ability of adenovirus to infect cells of the brain in vivo in two regions, the hippocampus and the substantia nigra (*9*). All injected animals expressed β-gal activity and β-gal protein, which were detected as early as 24 hours after inoculation and also in animals analyzed after 2 months. The diffusion of the virus was greater in the hippocampus than in the substantia nigra. Infected cells were found throughout the entire dorsal region of the hippocampus (Fig. 2), whereas in the substantia nigra the overall pattern of infection was restricted mainly to a medial-lateral orientation (Fig. 3D). This difference may reflect the propensity of the virus to spread through tissues that adhere loosely, such as the hippocampal fissure.

The extent of the infected area was correlated to the volume of viral solution administered. For instance, in rats killed 3 to 7 days after hippocampal inoculation, the infected area was 1 to 4 mm^3 for 3 to 5 μl of virus injected [10^{10} plaque-forming units (PFU) per milliliter]. Only minor differences in the distribution of the labeling were noted within the first week after inoculation (Fig. 2, A and B). At a longer time, however, the extent of the infected area was more restricted and the labeling was confined to the granule cell layer (Fig. 2C).

No cytotoxic effects in the infected animals were apparent. All recovered from the inoculation procedure without behavioral abnormalities. Examination of the virus-infected brains revealed no enlargement of the lateral ventricle or disruption of the normal anatomy of the structures. The only noticeable alteration was local tissue necrosis and reactive gliosis that were restricted to the injected sites. This phenomenon was largely a result of injection trauma because a similar alteration was observed in animals that had been injected with saline. Finally, analysis of hippocampal cells with Nissl staining showed no cell loss or evidence of cytolysis within the pyramidal or granule cell layers.

We then characterized the infected cell types. At early times (1 to 7 days), many of the β-gal–stained cells exhibited a morphology characteristic of microglial cells in both regions. These small cells had fine, highly branched processes extending radially from the cell body (Fig. 3, A to C). Their identification as microglial cells was confirmed by additional labeling with the antibody OX42, which is directed against type 3 complement receptors (*10*), and with B4-isolectin (*11*). Some of the infected cells were astrocytes, as demonstrated by double staining with the X-gal substrate and an antibody directed against GFAP.

We next determined whether neurons also were infected in both cerebral regions. In the substantia nigra, double-labeling experiments demonstrated coexpression of β-gal and immunoreactivity for tyrosine hydroxylase (TH), a classical marker of catecholaminergic neurons (Fig. 3D). About 50% of the β-gal–positive cells within the infected dopaminergic cell area were marked

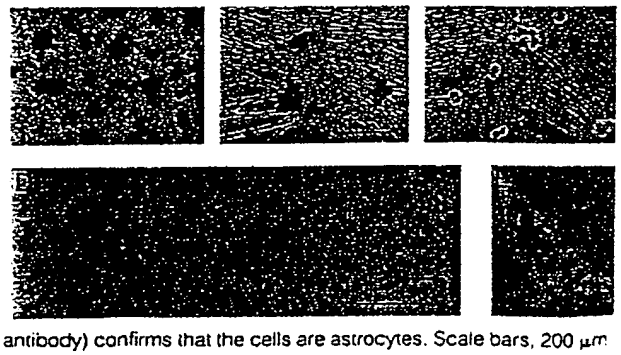

Fig. 1. Expression of β-gal in primary cultured cells after inoculation by adenovirus Ad.RSVβgal. (A and B) Virtually all the SCG neurons (*13*) expressed β-gal. (C) In the absence of the virus, no labeling was observed. (D) In enriched astroglial cultures (*14*), about two-thirds of the cells were labeled. (E) Additional staining with GFAP (Dakopatts, Glostrup, Denmark, 1:500 dilution, fluorescein-conjugated secondary antibody) confirms that the cells are astrocytes. Scale bars, 200 μm

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with a TH antibody, which thereby demonstrates that dopaminergic neurons were infected (Fig. 3, E to G). In the hippocampus, which is composed of segregated and laminated cellular subgroups, numerous β-gal–stained cells were unambiguously identified as neurons on the basis of morphological and anatomical characteristics (Fig. 3, H to J). Recognition of the cellular type was further facilitated because a few cells revealed a Golgi-like profile as a result of the diffusion of β-gal enzyme. These positively stained cells could be identified as pyramidal neurons, granule cells, and hilar interneurons in the pyramidal cell layer CA1, the granule cell layer, and the hilus of the dentate gyrus, respectively (Fig. 3, H to J).

In rats killed 1 and 2 months after inoculation, the distribution of β-gal–positive cells was more restricted than what had been observed at earlier times. Although microglial cells represented a large number of β-gal–expressing cells up to 1 week after injection into the hippocampus, their number decreased at longer post-infection times. Most of the labeled cells at 1 month were neurons of the stratum granulosum (Fig. 4). As determined in sections counterstained with cresyl violet, β-gal–positive cells were restricted to the granular layer, and no positive cells were seen in the innermost part of the layer that includes most of the basket cells and a few glial cells. The same pattern was also obtained at 2 months. This restriction in the pattern may reflect a change in the RSV LTR promoter activity; the activity of the RSV LTR promoter may be more

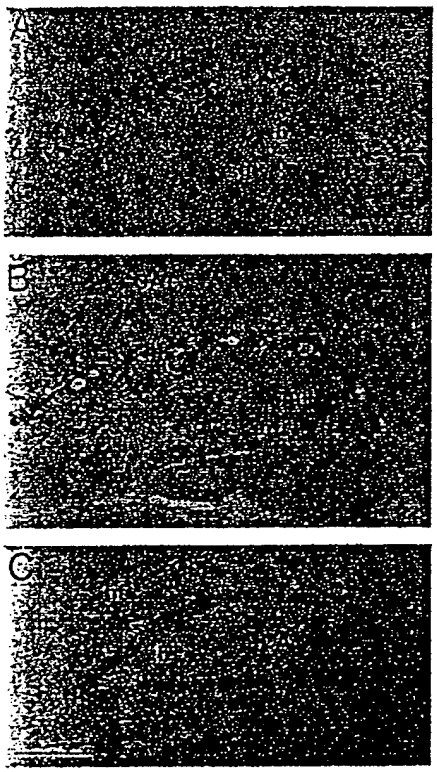

Fig. 2. General patterns of β-gal expression after unilateral intrahippocampal inoculation of the virus Ad.RSVβgal. (A) Staining with X-gal and fushin (Gurr, England) in a 40-μm-thick frontal section of the brain of a rat killed 24 hours after injection. (B) Immunohistochemical detection of β-gal 1 week after injection. The primary antibody was an affinity-purified rabbit immunoglobulin G fraction to β-gal (Cappel, Organon, West Chester, Pennsylvania, 1:800 dilution) that was then bound with a streptavidin-biotinylated peroxidase complex (Amersham) with diaminobenzidine as a chromogen, reinforced with nickel. (C) Distribution of β-gal–positive blue cells in the dentate gyrus of the hippocampus 1 month after injection. Counterstaining is shown in neutral red. Scale bar, 300 μm; h, hilus; hf, hippocampal fissure; ml, molecular layer; sg, stratum granulosum.

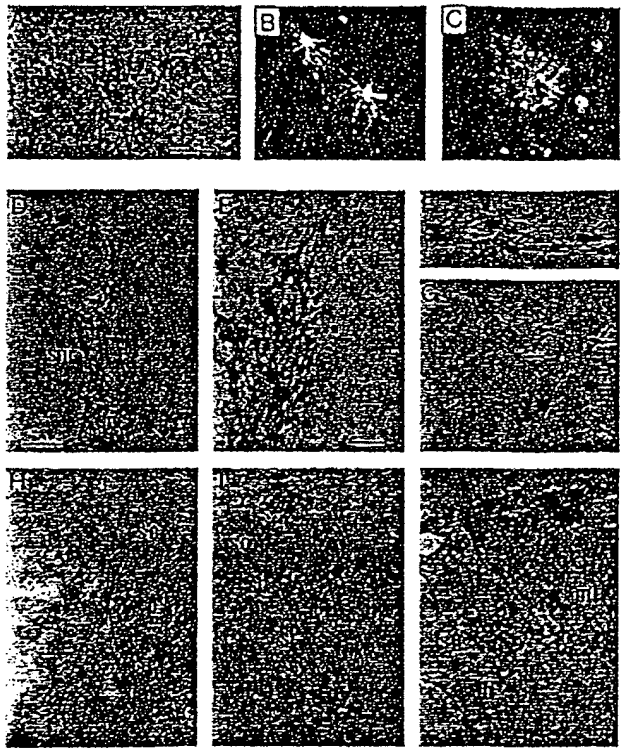

Fig. 3. Characterization of glial and neuronal cell types infected by direct in vivo inoculation of the adenovirus Ad.RSVβgal. (A to C) Immunohistochemical detection of β-gal expression in microglial cells 5 days after the injection into the hippocampus. Immunological reaction was processed with peroxidase reinforced with nickel (A) and with fluorescein-conjugated secondary antibody (B and C). (D to G) Sections across the substantia nigra. Three days after intranigral inoculation, a dense blue β-gal staining was detectable in a great number of nigral cells (D), most of which were also shown to be double-labeled with TH monoclonal antibodies (E to G) (Boehringer Mannheim, 1:200 dilution). Panel (E) is a higher magnification of the area with the highest density of β-gal–positive cells in (D). Arrows in (F) and (G) indicate cells double-stained for β-gal and TH. (H to J) Sections across the hippocampus. Pyramidal cells in CA1 (H and I) and granule cells in the dentate gyrus (J) are labeled. β-gal activity was revealed by immunohistochemistry as in (A). The cells in (D) to (G) were processed with histochemistry; in addition, the cells in (D) to (J) were labeled with antibodies. Scale bars: 30 μm in (A), 300 μm in (D), and 100 μm in (E) and (H). Abbreviations are as in Fig. 2 except for snc, substantia nigra pars compacta; snr, substantia nigra pars reticulata; sp, stratum pyramidale; so, stratum oriens; and sr, stratum radiatum.

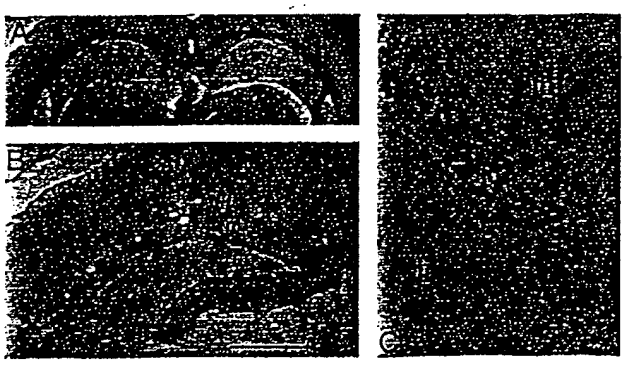

Fig. 4. Distribution of β-gal–positive cells in the dentate gyrus 1 month after Ad.RSVβgal inoculation. (A) Photomicrograph stained for β-gal expression with X-gal histochemistry. The cells that are stained blue were observed in the dentate gyrus of the injected left hippocampus. Scale bar, 1 mm. (B) Dentate localization of infected cells was confirmed by immunohistochemical β-gal detection (staining with peroxidase plus nickel). Scale bar, 300 μm. (C) High magnification view showing the large number of densely packed, β-gal–labeled cell nuclei in the granule cell layer of the dentate gyrus. Abbreviations are as in Fig. 3.

stable in neurons than in glial cells. Another possibility is that the virus may be transferred from one cell type to another, as has been described for rabies and herpes viruses (*12*).

The use of adenovirus vectors provides a method to study the function of cloned genes, which is complementary to that of transgenic animals. For instance, infection of the hippocampus would be useful for the study of integrated phenomena such as long-term potentiation. The possibility of selecting the time at which a particular gene is to be expressed is important when the expression of a transgene in early development is deleterious to the animal. In the context of degenerative diseases, it may also be possible to express neurotransmitters or growth factors locally as an alternative to the grafting of fetal cells.

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7. Histochemical staining was performed with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) as described [J. R. Sanes, J. L. Rubenstein, J. F. Nicolas, *EMBO J.* 5, 3133 (1986)].
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9. Seventeen male Wistar rats (10 weeks old) were stereotactically injected under deep anesthesia with 1 to 5 μl of media that contained highly purified virus (10^{10} PFU/ml) into either the hippocampus or the substantia nigra. Animals were killed 1, 2, 3, 5, 7, 30, and 60 days after inoculation. We detected β-gal activity in positive cells histochemically by using both the X-gal substrate and an antibody directed against the protein. The latter method is more sensitive and in some instances revealed fine cytoplasmic processes.
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13. The SCG were removed from 2-day-old Wistar rats, dissociated, plated on 16-mm collagen-coated dishes, and cultured as described (*7*). Cytosine arabinofuranoside (10 μM) was added during the first week of culture to prevent proliferation of ganglionic non-neuronal cells. After 6 days in culture, the cells were inoculated with 10^6 PFU of Ad.RSVβgal in culture medium or, as a control, exposed only to culture medium. Twenty-four hours later, the virus was removed, and the cells were maintained for 2 days in culture medium. After washing and paraformaldehyde fixation, β-gal–expressing cells were characterized with X-gal histochemistry.
14. Cells were plated in 35-mm-diameter plastic dishes and grown in supplemented Dulbecco's modified Eagle's medium for 5 days. The cells in each dish were then inoculated with 2 μl of the adenoviral solution (titer, 10^8 PFU/ml) for 24 hours. Histochemical staining was processed as in (*13*).
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CD40 Ligand Gene Defects Responsible for X-Linked Hyper-IgM Syndrome

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The ligand for CD40 (CD40L) is a membrane glycoprotein on activated T cells that induces B cell proliferation and immunoglobulin secretion. Abnormalities in the CD40L gene were associated with an X-linked immunodeficiency in humans [hyper-IgM (immunoglobulin M) syndrome]. This disease is characterized by elevated concentrations of serum IgM and decreased amounts of all other isotypes. CD40L complementary DNAs from three of four patients with this syndrome contained distinct point mutations. Recombinant expression of two of the mutant CD40L complementary DNAs resulted in proteins incapable of binding to CD40 and unable to induce proliferation or IgE secretion from normal B cells. Activated T cells from the four affected patients failed to express wild-type CD40L, although their B cells responded normally to wild-type CD40L. Thus, these CD40L defects lead to a T cell abnormality that results in the failure of patient B cells to undergo immunoglobulin class switching.

Human hyper-IgM immunodeficiency is a rare disorder characterized by normal or elevated serum concentrations of polyclonal IgM and markedly decreased concentrations of IgA, IgE, and IgG (*1*, *2*). Reports of X-linked, autosomal recessive, autosomal dominant, and acquired forms of the disorder indicate genetic heterogeneity and that several different pathologic mechanisms may be responsible (*2*, *3*). In the X-linked form of hyper-IgM syndrome, affected males usually experience the onset of recurrent infections in the first year of life. Affected males have normal numbers of circulating B and T lymphocytes, although lymph node hyperplasia with an absence of germinal centers is common (*1*, *2*). This condition is lethal in the absence of medical intervention; however, patients typically respond well to a maintenance therapy consisting of intravenous treatment with γ globulin.

The cellular abnormalities that underlie the various forms of hyper-IgM syndrome are unclear. Studies of patterns of X chromosome inactivation in obligate carrier females indicate a randomized pattern of X chromosome usage in either B or T lineage cells, which suggests that the defect does not alter maturation of these cells by cell autonomous mechanisms (*4*). Some studies have suggested that the affected phenotype is likely a result of B cell dysfunction insofar as patient B cells treated with polyclonal B cell activators, such as pokeweed mitogen, could not be induced to switch to IgG or IgA production (*5*). Other reports suggest that the

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Adenovirus VAI RNA Is Required for Efficient Translation of Viral mRNAs at Late Times after Infection

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Summary

Two adenovirus type 5 mutants were constructed to probe the function of the virus-encoded RNA polymerase III transcripts (VA RNAs). Each mutant fails to synthesize one of the two VA RNA species. The variant that does not produce the minor VAII species grows normally. The mutant that cannot synthesize the major VAI species grows more poorly than its parent. Analysis of the mutant's growth defect indicates that the adenovirus VAI RNA is required for efficient translation of viral mRNAs at late times after infection.

Introduction

The adenovirus (Ad) VA RNAs are small RNAs (about 160 nucleotides) synthesized in large amounts late after infection (Reich et al., 1966; Soderlund et al., 1976). The RNAs are encoded by two different genes, which are designated VAI and VAII (Mathews, 1975). The genes are located at about 30 map units on the Ad2 or Ad5 genome (Mathews, 1975; Pettersson and Philipson, 1975; Soderlund et al., 1966), and their nucleotide sequence has been delineated (Ohe and Weissman, 1970; Thimmappaya et al., 1979; Akusjarvi et al., 1980). The VA RNA genes are transcribed by RNA polymerase III (Weinmann et al., 1974; Soderlund et al., 1976) and contain intragenic transcriptional control regions (Fowlkes and Shenk, 1980; Guilfoyle and Weinmann, 1981). VAI RNA is made in much larger amounts at late times after infection than the VAII species (about 40:1). Some and perhaps all of the VA RNA molecules exist as ribonucleoprotein particles in association with at least one cellular protein antigen, which is recognized by the anti-La class of lupus sera (Lerner et al., 1981a, 1981b).

To probe the function of the VA RNAs, we have constructed two Ad5 variants, each of which fails to synthesize one of the VA species. The mutant that does not produce the minor VAII species grows normally. However, the mutant that cannot synthesize the major VAI species grows more poorly than its parent. Analysis of the mutant's growth defect leads us to conclude that VAI RNA is required for efficient translation of viral mRNAs at late times after infection.

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Results

Construction of Variants

We have previously constructed and characterized deletion mutations within the adenovirus VA RNA genes carried on recombinant plasmids (Fowlkes and Shenk, 1980). Several of the deletions destroyed the ability of the cloned genes to function as templates for RNA polymerase III in cell-free lysates. Two of these transcriptionally inactive VA RNA genes were chosen to be rebuilt into intact viral chromosomes, replacing the wild-type loci. One carries a deletion of 29 bp within the VAI RNA gene (pA2-*dl*4), and the other lacks 17 bp within the VAII coding region (pA2-*dl*8).

The protocol for reconstructing the plasmid-derived segments back into the viral chromosome is diagrammed in Figure 1. Small DNA segments carrying the VA-specific deletions were first transferred to plasmids that contained much larger segments of the viral genome. Intact viral chromosomes were then rebuilt via a three-fragment ligation. A 0–29 map unit fragment was prepared from *dl*313 viral DNA; the 29–46 map unit segment was from the recombinant plasmid carrying the VA deletion; and a 46–100 map unit fragment was obtained from *dl*324. The 313 and 324 deletions were included in the construction for technical purposes (discussed in the Experimental Procedures). The 324 deletion (79–85 map units) is located within early region 3, a transcription unit known to be nonessential for growth of adenovirus in cell culture. The 313 deletion lies within early region 1. Viruses carrying this deletion can be complemented by propagation in 293 cells (a human embryonic kidney cell line that contains and expresses the Ad5 early region 1; Graham et al., 1977). As a result, the 313 and 324 deletions will not affect the phenotypes of reconstituted viruses, provided they are studied in 293 cells.

Both viruses carrying VA-specific mutations are viable and can be propagated in 293 cell cultures. The mutant lacking a portion of its VAI RNA gene is designated *dl*330, and that lacking a segment of the VAII RNA gene is *dl*328 (Figure 2). A derivative of *dl*330 was prepared that does not contain the 313 deletion and is capable of growth in HeLa cells. This derivative is *dl*331, and is indistinguishable from *dl*330 in its VAI RNA–specific properties.

The Mutants Each Fail to Produce One VA RNA Species In Vivo

As expected, *dl*330 and 328 DNA failed to direct the synthesis of VAI and VAII RNA, respectively, when assayed in a cell-free polymerase III extract (Figure 2). To test that the mutations also prevented transcription in vivo, infected 293 cells were labeled with $^{32}PO_4^{3-}$ and their cytoplasmic RNAs were analyzed

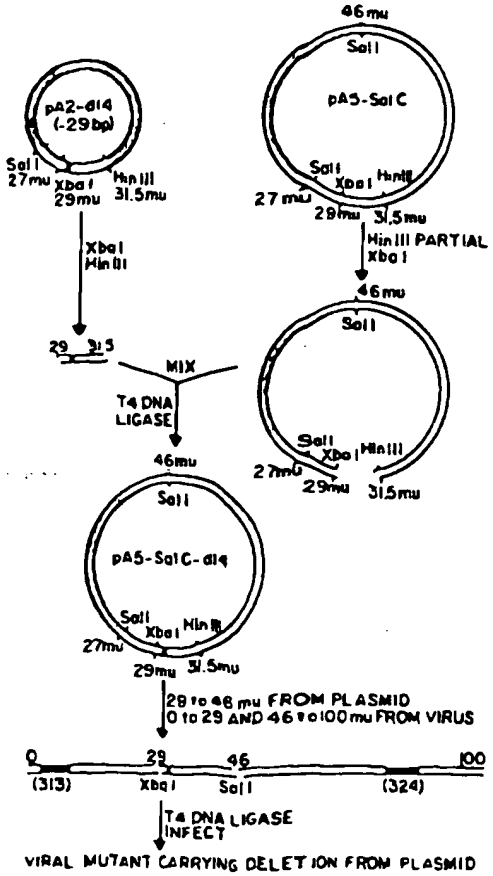

Figure 1. Diagram of the Protocol Used for Construction of Ad5 Variants Carrying Deletions within the VA RNA Coding Regions

The example pictured is the construction of *d*l330 (VAI$^-$/VAII$^+$). Shaded portions: pBR322 segments. Solid bands: deletions. Restriction endonuclease cleavage sites are positioned in Ad5 map units

(Figure 2). While cells infected with *d*l324 (isogenic to *d*l328 and 330 except at the VA locus, where it lacks 2 bp and fails to synthesize the VAI(A) species; Thimmappaya et al., 1979) contained large amounts of VAI RNA, none was evident in *d*l330-infected cells. Furthermore, *d*l330-infected cells contained greater than normal amounts of the VAII RNA. This is consistent with our earlier observation that a functional VAI gene competitively inhibits synthesis of the VAII species in vitro (Fowlkes and Shenk, 1980). It was not possible to detect VAII RNA synthesized in whole cells by any of the viruses. To circumvent this problem, nuclei were prepared from infected cells and incubated for a short period in the presence of α-^{32}P-UTP. VAII RNA was clearly detectable in nuclei from cells infected with *wt*300, *d*l324 or *d*l330 but not with *d*l328 (Figure 2). Thus the 328 and 330 deletions prevent transcription of the altered VA RNA genes both in vitro and in vivo.

Growth Characteristics of Viruses Carrying VA-Specific Mutations

The growth kinetics and yield of mutant and wild-type viruses were compared (Figure 3). The growth of

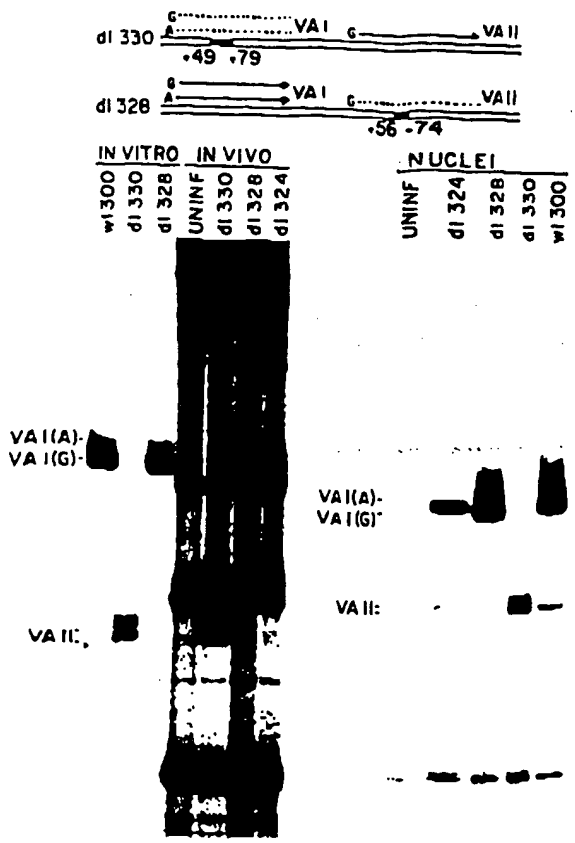

Figure 2. Electrophoretic Analysis of VA RNAs

In vitro transcription reactions were carried out as described by Fowlkes and Shenk (1980) using whole viral DNAs as template in vivo analyses were performed by infecting 293 cells with the indicated viruses at a multiplicity of 5 pfu/cell, labeling from 2-18 hr after infection with $^{32}PO_4^{3-}$ (200 μCi/ml) and extracting total cytoplasmic RNA. Nuclei were isolated (Weinmann et al., 1974) at 17 hr after infection and labeled for 20 min with α-^{32}P-UTP (200 μCi/ml) before total nuclear RNA was prepared. Electrophoresis was performed using a 6% polyacrylamide slab gel (0.6 mm thick, 40 cm long, containing 8 M urea in a Tris-borate buffer) for either 9 or 12 hr at 500 V. *d*l324 produces only the VAI(G) species due to a 2 bp deletion in the 5'-flanking region of the VAI transcription unit (Thimmappaya et al., 1979). The 330 and 328 deletions within the VAI and VAII RNA coding regions are diagrammed at the top. The deletions are located relative to the VA transcriptional start sites (A start for VAI), and the base pairs identified are the last present on either boundary of the deletions.

*d*l328 (VAI$^+$/VAII$^-$) is very similar to that of either *d*l324 (parental virus) or *wt*300 in 293 cells. In contrast, *d*l330 (VAI$^-$/VAII$^+$) grows more slowly and reaches a 20-fold reduced yield relative to the other viruses by day 5, when all infected cells have detached from the culture dish.

The Poor Growth of *d*l330 Is Due to a Lack of VAI RNA

It was important to establish that the reduced growth potential of *d*l330 resulted from the lack of VAI RNA. There is an open reading frame on the VAI RNA-coding DNA strand, and it was conceivable that the

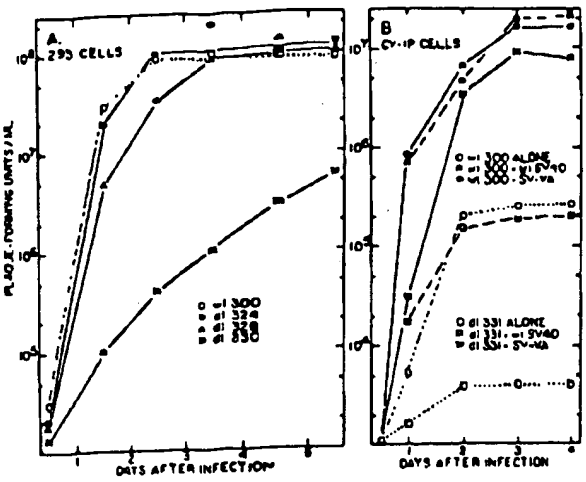

Figure 3 Growth Kinetics of Mutant and Wild-Type Viruses and Complementation of the *d*l331 (VAI⁻/VAII⁻) Mutation with an SV40 Recombinant Carrying the VAI RNA Gene

(A) To monitor growth kinetics, 293 cells were infected at a multiplicity of 3 pfu/cell, and the virus yield at the indicated times after infection was measured by plaque assay on 293 cells. (B) To demonstrate complementation, CV-1P cells were infected with SV40 or SV-VA at a multiplicity of 5 pfu/cell; 40 hr later the cells were superinfected with Ad5 (*wt*300 or *d*l331) at a multiplicity of 3 pfu/cell. Yields of Ad5 at the indicated times after Ad5 infection were measured by plaque assay on 293 cells.

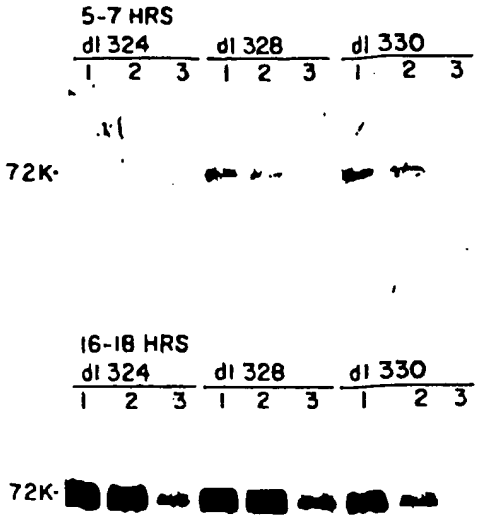

Figure 4 Electrophoretic Analysis of the Early Region 2A-Specific 72 kd Polypeptide Synthesized in 293 Cells after Infection with *d*l324 (VAI⁺/VAII⁺), *d*l328 (VAI⁺/VAII⁻) or *d*l330 (VAI⁻/VAII⁻)

Cells were infected at a multiplicity of 5 pfu/cell and labeled with ^{35}S-methionine (50 μCi/ml) 5–7 (top) or 16–18 (bottom) hr after infection. Extracts were prepared, and immunoprecipitations were performed using a monoclonal antibody (l-1; gift from A. Levine) specific for the adenovirus early region 2A 72 kd polypeptide. Accurate quantitation of 72 kd polypeptide levels was achieved by subjecting the supernatants generated by the first precipitation (lanes 1) to two additional cycles (lanes 2 and 3) of immunoprecipitation. Electrophoresis was carried out for 18 hr at 15 mA in a 15% polyacrylamide gel containing 0.1% SDS, as described by Sarnow et al. (1982).

330 deletion altered an as-yet-unidentified polypeptide. To rule out this possibility, we tested the ability of a recombinant SV40 virus carrying and expressing the Ad5 VAI RNA gene (designated SV-VA; Weinberger et al., 1981) to complement the *d*l330 defect (Figure 3). The experiment was performed in monkey kidney (CV-1P) cells to permit optimal growth of the SV40 recombinant. Wild-type Ad5 (*wt*300) grows poorly in monkey kidney cells. As has been known for many years, its poor growth is complemented by coinfection with SV40. Mutant *d*l331 (VAI⁻/VAII⁻, a derivative of *d*l330) also grows very poorly in monkey kidney cells. Wild-type SV40 only partially complements the *d*l331 growth defect, while coinfection with the SV-VA recombinant generates a near wild-type yield. The only adenovirus-specific gene product encoded by SV-VA is the VAI RNA. Thus we conclude that the *d*l330/331 growth defect results from the inability to synthesize VAI RNA, and this defect can be complemented in trans.

Viral mRNAs Are Translated Inefficiently at Late Times after Infection in *d*l330-Infected Cells

The *d*l330 (VAI⁻/VAII⁻) growth cycle was found to proceed normally through the production of late mRNAs. Polypeptide synthesis at late times after infection, however, was dramatically reduced. The following events were monitored.

Early Protein Synthesis and DNA Replication

Analysis of viral polypeptides produced at 5–7 hr after infection (E1B, 58 kd, data not shown; E2A, 72 kd, Figure 4) by immunoprecipitation indicated that they were synthesized at enhanced levels in 293 cells infected with *d*l330 as compared with *wt*300- or *d*l324-infected cells. This early perturbation is not due to altered VA RNA expression, since a derivative of *d*l330 that contains and expresses a functional VAI gene inserted in region E3 also synthesized enhanced levels of early polypeptides at 5–7 hr after infection (data not shown). Furthermore, altered early expression cannot be the cause of the late *d*l330 defect described below, since *d*l328 exhibits an identical early phenotype but no late defect (Figure 4).

Viral DNA replication was normal in *d*l330-infected 293 cells (Figure 5). In fact, it is our impression from several experiments that the onset of viral DNA replication occurs somewhat earlier in *d*l330- than in *wt*300- or *d*l324-infected cells. This may be due to enhanced early gene expression in *d*l330-infected cells. It is also clear that *d*l330 is capable of shutting off host-cell DNA synthesis (Figure 5).

Synthesis of mRNAs at Late Times after Infection

Figure 6A depicts a quantitative Northern-type analysis of cytoplasmic, poly(A)⁺ RNAs produced in *d*l330- or *d*l324-infected cells at 17 hr after infection. Identical RNA transfers were probed with ^{32}P-labeled DNAs corresponding to either a transcription unit expressed

at both early and late times after infection (E2A) or to a late family of mRNAs (L3). Mutant-infected cells contained about the same levels of E2A- and L3-specific RNAs as did cells infected with the parental virus. Similar results were obtained using DNA probes specific for two additional late mRNA families (L1 and L5; data not shonw).

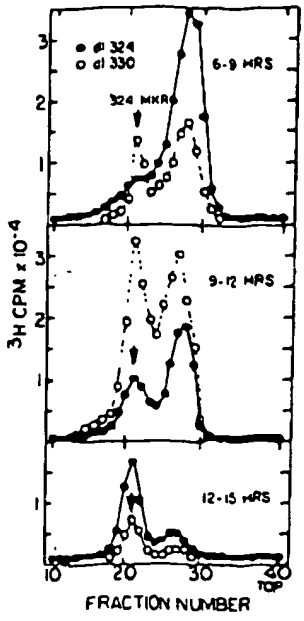

Figure 5 Equilibrium Density Centrifugation of DNAs Synthesized in 293 Cells Infected with *dl*324 (VAI$^-$/VAII$^-$) or 330 (VAI$^-$/VAII$^+$)

Cells were infected at a multiplicity of 5 pfu/cell and labeled with ^{3}H thymidine (10 μCi/ml) for the indicated time periods after infection. Total cellular DNA was prepared and analyzed by centrifugation. Arrows, the location of ^{32}P-labeled *dl*324 DNA, which was included as a marker (MKR).

Splicing of Late mRNAs

All of the mRNAs encoded within the major late transcription unit contain leader sequences encoded at 16.5, 19.5 and 26.5 map units joined to coding regions located between 30 and 90 map units. So, if the late species were not spliced, they would be much larger and would migrate more slowly when subjected to electrophoresis. The *dl*330-specific late mRNAs are most likely properly spliced, since they migrate identically to *dl*324-specific species (Figure 6A). To further establish that late RNAs encoded by *dl*330 are properly spliced, a primer-extension experiment was performed (Figure 6B). A small DNA fragment corresponding to the 5′-coding region of the major L3 mRNA was annealed to total poly(A)$^+$ RNA, and the hybridized primer was extended using reverse transcriptase. The length of the extended product was identical for *dl*324- and 330-specific RNAs. Thus the *dl*330-specific mRNA is properly processed to generate the characteristic late tripartite leader joined to the L3 coding region.

Capping and Polyadenylation of Late mRNAs

293 cells were infected with either *dl*324 or 330 and labeled 14–18 hr after infection with ^{3}H-adenosine. Cytoplasmic, poly(A)$^+$ RNA was prepared, and >80% of the labeled RNA was shown to be virus-specific. The relative amounts of label in type 1 cap structures and poly(A) tracts were determined (Table 1). *dl*324- and 330-specific RNAs were nearly indistinguishable, and we conclude that late viral mRNAs are properly capped and polyadenylated in the absence of VAI RNA.

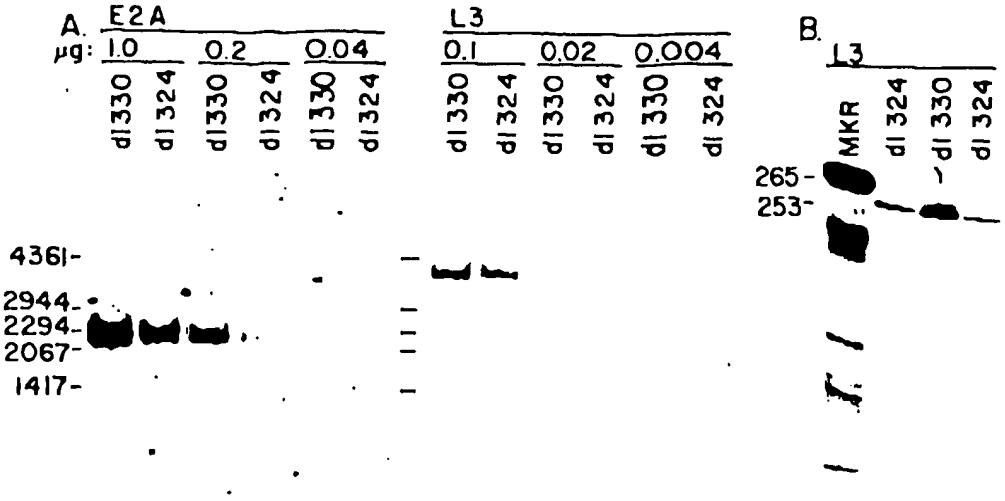

Figure 6 Analysis of Viral mRNA Species Present at Late Times after Infection in 293 Cells Infected with *dl*324 (VAI$^-$/VAII$^-$) or 330 (VAI$^-$/VAII$^+$)

(A) Northern-type analysis of viral mRNAs. Cytoplasmic, poly(A)$^+$ RNA was prepared from 293 cells at 17 hr after infection with either virus at a multiplicity of 5 pfu/cell. The indicated amounts (in micrograms) of RNAs were subjected to electrophoresis at 100 V for 4 hr in 1% agarose gels containing 6% formaldehyde (Rave et al., 1979). Plasmid DNA probes contained sequences specific for Ad2 early region 2A (E2A) or late region 3 (L3). (B) Primer-extension analysis of L3 mRNAs specific for hexon. The extended primer was subjected to electrophoresis for 5 hr at 800 V in an 8% polyacrylamide gel (0.3 mm thick, 40 cm long, containing 8 M urea in a Tris–borate buffer).

Synthesis of Polypeptides at Late Times after Infection

Infected 293 cells were labeled with ^{35}S-methionine 16–20 or 24–48 hr after infection, and polypeptides were analyzed by electrophoresis (Figure 7). *d*l330-infected cells contained reduced levels (about 8-fold) of all late polypeptides as compared with cells infected with either *d*l324 or 328. Expression of two early polypeptides (E1B, 58 kd, data not shown; E2A, 72 kd, Figure 4) was also monitored and found to be reduced (8 to 10 fold) in *d*l331-infected cells at late times after infection. The low level of *d*l330-specific polypeptides appears to be due to a reduced rate of protein synthesis (as opposed to an increased rate of degradation), since the reduction is evident during labeling periods as short as 2 min. Furthermore, analysis of a *d*l330-infected culture by immunofluorescence indicates that all cells contain similar levels of properly localized late polypeptides (as opposed to different levels in different cells) (data not shown).

The experiment depicted in Figure 7 also indicates that host-cell protein synthesis is shut off subsequent to infection with the VAI$^-$ mutant (compare lanes MOCK with lanes *d*l330). The VAII$^-$ mutant also shuts off host-cell synthesis (not apparent in this experiment, since *d*l328 was inadvertently used at too low a multiplicity to infect all cells).

Late *d*l330 mRNAs Are Efficiently Translated in Cell-Free Reticulocyte Lysates

Total, poly(A)$^+$ RNA was prepared from both *d*l324 (VAI$^+$/VAII$^+$)- and 330 (VAI$^-$/VAII$^-$)-infected 293 cells and used to program mRNA-dependent, rabbit reticulocyte lysates. The response to *d*l324- or 330-specific RNAs was essentially identical, and was dependent on the concentration of added RNA (Figure 8). There are several relatively minor differences between the translation products of the two RNA populations. The reproducibility of this observation and the identity of the altered polypeptides are under investigation. It seems clear, nevertheless, that *d*l330-infected cells contain normal quantities of functional late mRNAs, but they are not efficiently translated.

Table 1 *d*l330 Late mRNAs Are Properly Capped and Polyadenylated

	Percentage of Total ^{3}H-Adenosine[a]	
	*d*l324	*d*l330
Ad5-specific RNA[b]	>80	>80
Type 1 cap[c]		
m^{7}GpppA^m	0.039	0.043
m^{7}Gpppm^{6}A^m	0.032	0.042
Poly(A)[d]	18.23	18.90

[a] Cells were labeled with ^{3}H-adenosine (250 μCi/ml) 14–18 hr after infection.

[b] Total cytoplasmic, poly(A)$^+$ RNA was prepared. ^{3}H-labeled RNA was completely resistant to S1 endonuclease after annealing in the presence of excess viral DNA. RNA annealed in the absence of DNA was 20% S1-endonuclease-resistant.

[c] RNA was digested with T2 RNAase, P1 nuclease and bacterial alkaline phosphatase. Products were analyzed by reversed-phase high-pressure liquid chromatography.

[d] RNA was digested with T1 and pancreatic RNAases. Products were analyzed by electrophoresis on a 6% polyacrylamide gel.

Discussion

Cells infected with *d*l330 or 331 (VA$^-$/VAII$^+$) contain normal levels of both early and late viral mRNAs (Figure 6). These RNAs appear to be properly capped, spliced and polyadenylated (Table 1; Figure 6); cellular fractionation indicates they are localized in the cytoplasm of infected cells; and they are efficiently translated in vitro (Figure 8). However, *d*l330- or 331-infected cells produce reduced levels of both early and late polypeptides at late times after infection (Figures 4 and 7). Thus we conclude that VAI RNA is required for efficient translation of viral mRNAs at late times after infection.

VAI RNA may also be required for translation of adeno-associated virus (AAV) mRNAs. Janik et al. (1981) have concluded that VA RNA is one of several adenovirus gene products required for AAV growth. Furthermore, Jay et al. (1981) have found that H5*ts*125 (which carries a single-base-pair change in the L2, 72 kd polypeptide; Ginsberg et al., 1974; Kruijer et al., 1981) fails to complement AAV at the

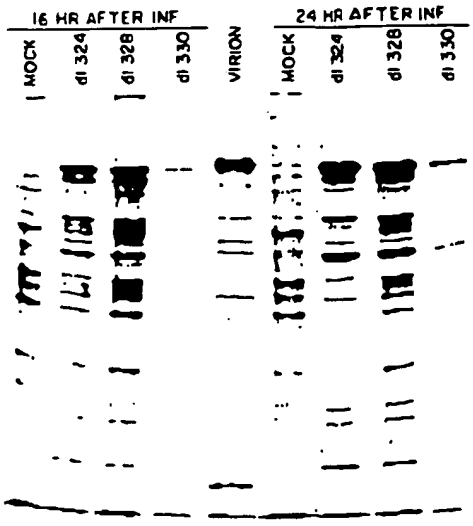

Figure 7. Electrophoretic Analysis of Polypeptides Synthesized in 293 Cells at Late Times after Infection with *d*l324 (VAI$^+$/VAII$^+$), *d*l328 (VAI$^+$/VA$^+$) or *d*l330 (VAI$^-$/VAII$^+$)

Cells were infected at a multiplicity of 5 plu/cell (except with *d*l328, which was used at 1 plu/ml) and labeled with ^{35}S-methionine (20 μCi/ml) 16–20 or 24–28 hr after infection. Extracts were prepared from labeled, infected cells or from mock-infected cells (lanes MOCK) and electrophoresis was carried out as described in the legend to Figure 4. (Lane VIRION) Labeled virion proteins, included as size markers.

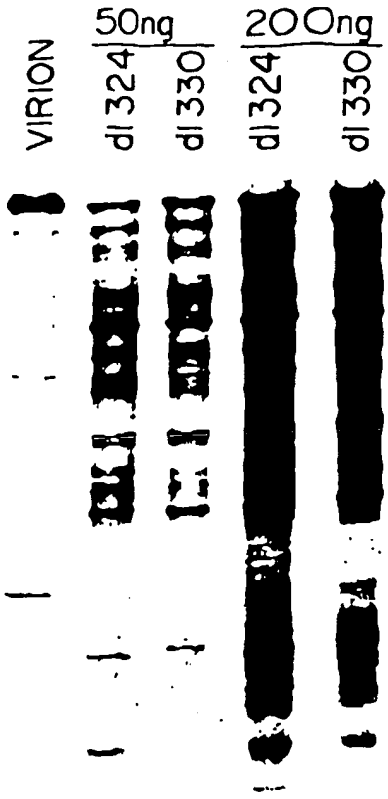

Figure 8. Electrophoretic Analysis of Polypeptides Synthesized in Rabbit Reticulocyte Lysates Programmed with *dl*324 (VAI$^-$/VAII$^+$) or *dl*330 (VAI$^-$/VAII$^-$) Poly(A)$^-$ RNAs

293 cells were infected at a multiplicity of 5 pfu/cell, and total cytoplasmic, poly(A)$^+$ RNA was isolated at 17 hr after infection. The RNA (50 or 200 ng) was translated in mRNA-dependent reticulocyte lysates as described by Pelham and Jackson (1976). Electrophoresis was carried out as described for Figure 7. (Lane VIRION) Labeled virion proteins, included as size markers.

nonpermissive temperature. Infected cells contain normal levels of AAV-specific mRNAs, but synthesis of the AAV structural polypeptides is reduced 50-fold. Since at the nonpermissive temperature H5*ts*125 fails to replicate its DNA (Ginsberg et al., 1974) and produces little VA RNA (Jones and Shenk, 1979b), the translational defect could well result from a lack of VAI RNA.

Like adenoviruses, Epstein-Barr virus encodes two small polymerase III transcripts called EBER1 and EBER2 (Lerner et al., 1981a; Rosa et al., 1981). These RNAs might also function at the level of translation. We are currently testing whether they can functionally substitute for the VA species.

Do VAI and VAII RNAs perform the same function? The two RNAs exhibit scattered homologies amounting to about 60% of their sequence (Akusjarvi et al., 1980). If their function is identical, one could suggest that *dl*328 (VAI$^+$/VAII$^-$) grows normally, since it still produces large amounts of the major VAI species and lacks only the minor RNA. Similarly, the poor growth of *dl*330 would be attributed to its inability to produce sufficient quantities of the VAII RNA to offset the loss of the VAI species. An alternative interpretation of our data could suggest that the VAII RNA functions poorly or not at all (it is nonessential and cannot functionally compensate for the lack of the VAI species). Since the functional status of the VAII species is unclear, we cannot yet say whether VAI RNA is absolutely required or merely enhances translation at late times after infection. We also cannot rule out the possibility that the VA RNAs perform additional functions during the viral growth cycle. To address these questions, we are currently attempting to construct and propagate a double mutant (VAI$^-$/VAII$^-$).

How does VAI RNA function in translation? So far, we have established that the rate of nascent polypeptide elongation at late times after infection is the same in *dl*331- and *wt*300-infected cells. Polysomes are smaller (that is, they contain fewer ribosomes per mRNA) at late times after infection in mutant than in wild-type-infected cells, and *dl*331-infected cells contain large amounts of free, 74S ribosomes (our unpublished data). These observations suggest a role for VAI RNA during initiation of translation. The mechanism by which VAI RNA facilitates initiation is unclear. Conceivably, the VA ribonucleoproteins (RNPs) serve to properly localize viral mRNPs in the cytoplasmic compartment, perhaps by attaching them to the cellular cytoskeleton, where they can be actively translated (Cervera et al., 1981) and/or to cellular structures that not only facilitate initiation but also allow proper cellular localization of the newly synthesized polypeptides. Alternatively, the particles may direct one or more initiation factors to viral mRNAs. Both of these models are attractive because they provide a rationale for the very large amount of VA RNAs produced in infected cells. In the first case, the RNPs perform a structural role; in the second, they outcompete, by virtue of their numbers, presumptive cellular RNPs, which serve a similar role in cellular protein synthesis.

Do small RNAs play a role in the initiation of translocation on cellular mRNAs? It is possible that VA RNAs serve a function required only in virus-infected cells. For example, infection by a variety of viruses is known to alter permeability of the cell membrane, causing an intracellular imbalance in small components such as ions, thiol compounds and ATP (see, for example, Carrasco, 1978; Fernández-Puentes and Carrasco, 1980; Ramabhadran and Thach, 1981). Thus VA RNAs could be required for efficient translation in an altered cellular environment. In a similar vein, VA RNAs could counteract a cellular antiviral response at late times after infection. Alternatively, the adenovirus VA RNAs may have counterparts that function during cellular translation. Animal cells contain a variety of small, cytoplasmic RNAs (see, for example, Eliceiri, 1974; Zieve and Penman, 1976; Jelinek and Leinwand, 1978; Weiner, 1980). The

number of RNA species appears quite large, given the recent demonstration that hamster Alu-equivalent interspersed-repetitive DNA sequences are transcribed in vivo (Haynes and Jelinek, 1981) and the likely possibility that many members of the human Alu family (Houck et al., 1979) are also expressed (as is the case in RNA polymerase III cell-free extracts: Duncan et al., 1979). Consistent with a role in translation, small RNAs have been reported either to base-pair to poly(A)$^+$ RNA (4.5S; Jelinek and Leinwand, 1978; VA can also bind to viral mRNAs: Mathews, 1980) or to be polysome-associated (7S: Walker et al., 1974; Gunning et al., 1981). Perhaps there are a variety of small, cytoplasmic RNAs that facilitate translation of broad classes of mRNAs. Conceivably, these small RNAs play a role in certain types of translational control. The heat-shock response is one example in which particular mRNAs are preferentially translated (Ashburner and Bonner, 1979; Scott and Pardue, 1981; Bienz and Gurdon, 1982). In fact, McCormick and Penman (1969) have inferred the existence of an RNA that promotes the initiation of peptide synthesis in HeLa cells subjected to heat shock. Translational control has also been described in a variety of developmental systems, including the slime mold (Alton and Lodish, 1977), surf clam (Rosenthal et al., 1980) starfish (Rosenthal et al., 1982) and mouse (Cascio and Wassarman, 1982). Although small RNAs have not yet been implicated, they could play central roles in these and other processes that regulate protein synthesis.

Experimental Procedures

Plasmids, Viruses, Cells and Enzymes

The recombinant plasmids pA2-*dl*4 and pA2-*dl*8 (Fowlkes and Shenk, 1980) contain a 28.5–31.5 map unit Ad2 DNA segment inserted between the Sal I and Hind III cleavage sites of pBR322 (Bolivar et al., 1977). pA2-*dl*4 lacks 29 bp at approximately 29.5 map units within the VAI RNA coding region (+49 to +78 relative to the VAI(A) transcriptional start site), and pA2-*dl*8 lacks 17 bp within the VAII RNA coding region (+56 to +74 relative to the VAII start site). pA5-SalC contains the Ad5 Sal I C fragment (27–46 map units) inserted at the pBR322 Sal I cleavage site. Plasmids were propagated in Escherichia coli strain HB101.

Wild-type Ad5 (H5*wt*300) is a plaque-purified derivative of a stock originally obtained from H. Ginsberg. H5*dl*324 contains the 313 deletion (3.8–10.2 map units, Jones and Shenk, 1979a) and the 309 deletion (−2 bp within the Xba I cleavage site at 29 map units; Thimmappaya et al., 1979), and it lacks the 79–84 map unit Xba I D fragment (referred to as the 324 deletion). H5*dl*328 and 330 are isogenic to H5*dl*324 except within the segment bounded by the 29 map unit Xba I site and 31.5 map unit Hind III cleavage site. This region has been replaced by pA2-*dl*8 sequences in *dl*328 (−17 bp within VAII) and pA2-*dl*4 sequences in *dl*330 (−28 bp within VAI). The construction of these mutants is diagrammed in Figure 1, and their VA-specific alterations are depicted in Figure 2. The VA mutations were originally constructed in a background containing the 313 and 324 deletions because we anticipated the mutants might be nonviable. The additional deletions form part of a complementation scheme. Since the mutants are viable, the additional deletions serve no purpose. The 324 deletion has no effect on virus growth in cell culture, but the 313 deletion confers a host-range phenotype on the viruses (Jones and Shenk, 1979a). As a result, *dl*324, 328 and 330 grow normally on 293 cells, which contain and express the early region 1 segment that they lack, but they are defective for growth in HeLa cells. H5*dl*331 was derived by marker rescue of *dl*330 for growth in HeLa cells, and it no longer contains the 313 deletion. H5*dl*330 in 293 cells and H5*dl*331 in HeLa cells are used interchangeably throughout this report.

Wild-type SV40 (*wt*830) is a plaque-purified derivative of the SV5 strain described by Takemoto et al. (1966). SV-VA is a recombinant SV40 that carries the Ad5 VAI RNA gene within its late coding region, and it is propagated using tsA58 as a helper virus (Weinberger et al., 1981).

The 293 cell line (Ad5-transformed human embryo cells; Graham et al., 1977), HeLa cells (obtained from J. Williams) and CV-1P cells (monkey kidney cells, Mertz and Berg, 1974) were maintained in Dulbecco's modified minimal essential medium containing 10% calf serum.

All restriction endonucleases were purchased from New England BioLabs. Polynucleotide kinase and DNA ligase from T4-infected E. coli and T1, T2 and pancreatic ribonucleases were from P-L Biochemicals. P1 nuclease was obtained from Yamasa Shoyu Co. S1 endonuclease was from Sigma, and bacterial alkaline phosphatase was from Worthington Biochemicals. Avian myeloblastosis virus reverse transcriptase was from J. W. Beard. Reaction conditions and units were as specified by the vendors.

RNA Preparation and Analysis

RNA was isolated from cells at 16 or 17 hr after infection at a multiplicity of 5 pfu/cell. A cytoplasmic fraction was prepared by suspending cells in isotonic buffer (10 mM Tris–HCl [pH 7.8], 1.5 mM $MgCl_2$, 150 mM NaCl), and Nonidet P-40 was added to a concentration of 0.6% after the mixture was cooled to 4°C. The mixture was held on ice for 10 min, then mixed by vortex for 10 sec. The nuclei were pelleted by centrifugation, and the supernatant was the cytoplasmic fraction. Cytoplasmic RNA was prepared by mixing this fraction with 3 volumes of 100 mM Tris–HCl (pH 9), extracting twice at room temperature with phenol (pH 9)–chloroform–isoamyl alcohol, 50:50:1 (v/v) and once with chloroform–isoamyl alcohol, 49:1 (v/v), and precipitating with 2 volumes of ethanol. Poly(A)$^+$ RNA was isolated by chromatography on oligo(dT)-cellulose.

Northern-type analysis (Alwine et al., 1977) of cytoplasmic, poly(A)$^+$ RNA was performed using nitrocellulose paper for RNA transfer according to the procedure of Thomas (1980). Primer-extension analysis of hexon-specific (L3) mRNAs employed the procedure described by Akusjarvi and Pettersson (1978). A 46 nucleotide (Taq I–Alu I) fragment specific for the 5′ coding region of the hexon mRNA was used as primer. To analyze cap structures, ^{3}H-adenosine-labeled, poly(A)$^+$ RNA was hydrolyzed with 50 U/ml T2 RNAase and 50 μg/ml P1 nuclease in 10 mM sodium acetate (pH 5) for 6 hr at 37°C. Ammonium hydroxide was added to a concentration of 10 mM, increasing the pH to 9, and 12 U/ml bacterial alkaline phosphatase was added for an additional 45 min at 37°C. Then the solution was evaporated to dryness and redissolved in 0.3% acetonitrile in 1 M ammonium formate. The products of hydrolysis were then resolved by reversed-phase high-pressure liquid chromatography using an Altex Ultrasphere octadecyl silane column (4.6 mm internal diameter × 25 cm) and a mobile phase containing increasing amounts of acetonitrile in 1 M ammonium formate (Albers et al., 1981). To analyze the poly(A) segment of mRNAs, ^{3}H-adenosine-labeled, poly(A)$^+$ RNA was hydrolyzed with 100 U/ml T1 and 1 μg/ml pancreatic RNAases in 0.2 M NaCl, 10 mM Tris–HCl (pH 7.4), 10 mM EDTA for 30 min at 37°C. The hydrolyzed RNA was subjected to electrophoresis on a 6% polyacrylamide gel. The position to which the poly(A) segments migrated was identified by fluorography (Laskey and Mills, 1975), they were cut from the gel and the radioactivity was measured.

Analysis of Polypeptides

In vivo experiments were performed by infecting 293 cells at a multiplicity of 5 pfu/cell and labeling with ^{35}S-methionine as indicated in the figure legends. Preparation of cellular extracts, immunoprecipitations and SDS-polyacrylamide gel electrophoresis were carried out as described by Sarnow et al. (1982). For in vitro analyses, cytoplas-

mic, poly(A)$^+$ RNA was prepared from infected cells as described above and used to program mRNA-dependent reticulocyte lysates as described by Pelham and Jackson (1976).

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